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(54) **Preparation of diagnostic agents**

Verfahren zur Herstellung von diagnostischen Mitteln

Préparation d'agents diagnostiques

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- **JOURNAL OF CONTROLLED RELEASE, vol.16, 1991 pages 279 - 289 ELSEVIER SCIENCE PUBLISHERS B.V.; Y. KAWASHIMA ET AL. 'Preparation of multiple unit hollow microsperes (microballoons) with acrylic resin containing tranilast and their drug release characteristics (in vitro) and floating behavior (in vivo)'**

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Description

[0001] The present invention relates to the preparation of diagnostic agents comprising hollow proteinaceous microcapsules used to enhance ultrasound imaging.

[0002] The fact that air bubbles in the body can be used for echocardiography has been known for some time. Bubble-containing liquids can be injected into the bloodstream for this purpose (see Ophir *et al* (1980) *"Ultrasonic Imaging"* 2, 67-77, who stabilised bubbles in a collagen membrane, US-A-4 446 442 (Schering) and EP-A-131 540 (Schering)) and EP-A-224934 and EP-A-324938 disclose the use of bubbles prepared by sonicating an albumin solution. However, the size distribution of the bubbles is apparently uncontrollable and the bubbles disappear when subjected to pressure experienced in the left ventricle (Shapiro *et al* (1990) *J. Am. Coll. Cardiology,* **16(7)**, 1603-1607).

[0003] EP-A-52575 discloses, for the same purpose, solid particles which have gas entrained in them, the gas being released from the particles in the bloodstream.

[0004] EP 458 745 (Sintetica) discloses a process of preparing air- or gas-filled microballoons by interfacial polymerisation of synthetic polymers such as polylactides and polyglycolides. WO 91/12823 (Delta) discloses a similar process using albumin. Wheatley *et al* (1990) *Biomaterials* **11**, 713-717, discloses ionotropic gelation of alginate to form microbubbles over 30 μm in diameter. WO-A-91/09629 discloses liposomes for use as ultrasound contrast agents.

[0005] Przyborowski *et al* (1982), *Eur.J. Nucl. Med.* **7**, 71-72, discloses the preparation of human serum albumin (HSA) microspheres by spray-drying, for radiolabelling and subsequent use in scintigraphic imaging of the lung. The microspheres were not said to be hollow and, in our repetition of the work, only solid microspheres were produced. Unless the particles are hollow, they are unsuitable for echocardiography. Furthermore, it was necessary in the process of Przyborowski *et al* to remove undenatured albumin from the microspheres, and a wide size range of microspheres was apparently obtained, as a further sieving step was necessary.

[0006] The Przyborowski *et al* article refers to two earlier disclosures of methods of obtaining albumin particles for lung scintigraphy. Aldrich & Johnston (1974), *Int. J. Appl. Rad. Isot.* **25**, 15-18, discloses the use of a spinning disc to generate 3-70 μm diameter particles which are then denatured in hot oil. The oil is removed and the particles labelled with radioisotopes. Raju *et al* (1978), *Isotopenpraxis* **14(2)**, 57-61, used the same spinning disc technique but denatured the albumin by simply heating the particles. In neither case were hollow microcapsules mentioned.

[0007] According to the present invention, a process for preparing hollow microcapsules, comprises atomising a solution or dispersion of a wall-forming protein or polysaccharide material in a liquid carrier into a gas, to obtain the hollow microcapsules by evaporation of the liquid carrier, substantially without reducing the water-solubility of at least the outside of the microcapsules.

[0008] According to a further aspect of the invention, microcapsules that are obtainable by a process of the invention, have a size such that more than 30% have a diameter within a 2 μm range and at least 90% have a diameter of 1 to 8 μm.

[0009] The wall-forming material and process conditions should be so chosen that the product is sufficiently non-toxic and non-immunogenic in use. The wall-forming material may be hydrophilic and biodegradable. Preferably, it is a protein of which examples are globulins and, preferably, gelatin, collagen and serum albumins. Such proteins are preferably of human origin (i.e. derived from humans or corresponding in structure to the human protein). Most preferably, it is human serum albumin (HA) derived from blood donations or, ideally, from the fermentation of microorganisms (including cell lines) which have been transformed or transfected to express HA.

[0010] Techniques for expressing HA (which term includes analogues and fragments of human albumin, for example those of EP-A-322094, and polymers of monomeric albumin) are disclosed in, for example, EP-A-201239 and EP-A-286424. "Analogues and fragments" of HA include all polypeptides (i) which are capable of forming a microcapsule in the process of the invention and (ii) of which a continuous region of at least 50% (preferably at least 75%, 80%, 90% or 95%) of the amino acid sequence is at least 80% homologous (preferably at least 90%, 95% or 99% homologous) with a continuous region of at least 50% (preferably 75%, 80%, 90% or 95%) of human albumin. HA which is produced by recombinant DNA techniques is particularly preferred. Thus, the HA may be produced by expressing an HA-encoding nucleotide sequence in yeast or in another microorganism and purifying the product, as is known in the art. Such material lacks the fatty acids associated with serum-derived material. Preferably, the HA is substantially free of fatty acids; ie it contains less than 1% of the fatty acid level of serum-derived material. Preferably, fatty acid is undetectable in the HA.

[0011] In the following description of preferred embodiments, the term "protein" is used since this is what we prefer but it is to be understood that other biocompatible wall-forming materials can be used, as discussed above.

[0012] The protein solution or dispersion is preferably 0.1 to 50% w/v, more preferably about 5.0 - 25.0% protein, particularly when the protein is albumin. About 20% is optimal. Mixtures of wall-forming materials may be used, in which case the percentages in the last two sentences refer to the total content of wall-forming material.

[0013] The preparation to be sprayed may contain substances other than the wall-forming material and solvent or carrier liquid. Thus, the aqueous phase may contain 1-20% by weight of water-soluble hydrophilic compounds like sugars and polymers as stabilizers, eg polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), polyethylene glycol (PEG),

gelatin, polyglutamic acid and polysaccharides such as starch, dextran, agar, xanthan and the like. Similar aqueous phases can be used as the carrier liquid in which the final microsphere product is suspended before use. Emulsifiers may be used (0.1-5% by weight) including most physiologically acceptable emulsifiers, for instance egg lecithin or soya bean lecithin, or synthetic lecithins such as saturated synthetic lecithins, for example, dimyristoyl phosphatidyl choline, dipalmitoyl phosphatidyl choline or distearoyl phosphatidyl choline or unsaturated synthetic lecithins, such as dioleyl phosphatidyl choline or dilinoleyl phosphatidyl choline. Emulsifiers also include surfactants such as free fatty acids, esters of fatty acids with polyoxyalkylene compounds like polyoxypropylene glycol and polyoxyethylene glycol; ethers of fatty alcohols with polyoxyalkylene glycols; esters of fatty acids with polyoxyalkylated sorbitan; soaps; glycerol-polyalkylene stearate; glycerol-polyoxyethylene ricinoleate; homo- and copolymers of polyalkylene glycols; polyethoxylated soya-oil and castor oil as well as hydrogenated derivatives; ethers and esters of sucrose or other carbohydrates with fatty acids, fatty alcohols, these being optionally polyoxyalkylated; mono-, di- and triglycerides of saturated or unsaturated fatty acids, glycerides or soya-oil and sucrose.

[0014] Additives can be incorporated into the wall of the microspheres to modify the physical properties such as dispersibility, elasticity and water permeability.

[0015] Among the useful additives, one may cite compounds which can "hydrophobize" the wall in order to decrease water permeability, such as fats, waxes and high molecular-weight hydrocarbons. Additives which improve dispersibility of the microspheres in the injectable liquid-carrier are amphipathic compounds like the phospholipids; they also increase water permeability and rate of biodegradability.

[0016] Additives which increase wall elasticity are the plasticizers like isopropyl myristate and the like. Also, very useful additives are constituted by polymers akin to that of the wall itself but with relatively low molecular weight. For instance the properties of the wall can be modified advantageously (enhanced softness and biodegradability) by incorporating, as additives, low molecular weight (1000 to 15,000 Dalton) polyglycolides or polylactides. Also polyethylene glycol of moderate to low Mw (eg PEG 2000) is a useful softening additive.

[0017] The quantity of additives to be incorporated in the wall is extremely variable and depends on the needs. In some cases no additive is used at all; in other cases amounts of additives which may reach about 20% by weight of the wall are possible.

[0018] The protein solution or dispersion (preferably solution), referred to hereinafter as the "protein preparation", is atomised and spray-dried by any suitable technique which results in discrete microcapsules of 1.00 - 50.0 µm diameter. These figures refer to at least 90% of the population of microcapsules, the diameter being measured with a Coulter Master Sizer II. The term "microcapsules" means hollow particles enclosing a space, which space is filled with a gas or vapour but not with any solid materials. Honeycombed particles resembling the confectionery sold in the UK as "Maltesers" (Regd TM) are not formed. It is not necessary for the space to be totally enclosed (although this is preferred) and it is not necessary for the microcapsules to be precisely spherical, although they are generally spherical. If the microcapsules are not spherical, then the diameters referred to above relate to the diameter of a corresponding spherical microcapsule having the same mass and enclosing the same volume of hollow space as the non-spherical microcapsule.

[0019] The atomising comprises forming an aerosol of the protein preparation by, for example, forcing the preparation through at least one orifice under pressure into, or by using a centrifugal atomizer in a chamber of warm air or other inert gas. The chamber should be big enough for the largest ejected drops not to strike the walls before drying. The gas or vapour in the chamber is clean (ie preferably sterile and pyrogen-free) and non-toxic when administered into the bloodstream in the amounts concomitant with administration of the microcapsules in echocardiography. The rate of evaporation of the liquid from the protein preparation should be sufficiently high to form hollow microcapsules but not so high as to burst the microcapsules. The rate of evaporation may be controlled by varying the gas flow rate, concentration of protein in the protein preparation, nature of liquid carrier, feed rate of the solution and, most importantly, the temperature of the gas encountered by the aerosol. With an albumin concentration of 15-25% in water, an inlet gas temperature of at least about 100°C, preferably at least 110°C, is generally sufficient to ensure hollowness and the temperature may be as high as 250°C without the capsules bursting. About 180-240°C, preferably about 210-230°C and most preferably about 220°C, is optimal, at least for albumin. Since the temperature of the gas encountered by the aerosol will depend also on the rate at which the aerosol is delivered and on the liquid content of the protein preparation, the outlet temperature may be monitored to ensure an adequate temperature in the chamber. An outlet temperature of 40-150°C has been found to be suitable. Apart from this factor, however, controlling the flow rate has not been found to be as useful as controlling the other parameters.

[0020] The microcapsules comprise typically 96-98% monomeric HA and have a limited *in vivo* life time for ultrasound imaging. They may, however, be used for ultrasound imaging, or they may be stored and transported before the second step of the two step process described in the parent Application is carried out.

[0021] It has been found that the process of the invention can be controlled in order to obtain microspheres with desired characteristics. Thus, the pressure at which the protein solution is supplied to the spray nozzle may be varied, for example from 1.0-10.0 x 10^5 Pa, preferably 2.0-6.0 x 10^5 Pa and most preferably about 5 x 10^5 Pa. Other parameters

may be varied as disclosed above and below. In this way, novel microspheres may be obtained.

[0022] A further aspect of the invention provides hollow microspheres in which more than 30%, preferably more than 40%, 50%, or 60%, of the microspheres have a diameter within a 2 μm range and at least 90%, preferably at least 95% or 99%, have a diameter within the range 1.0-8.0 μm.

[0023] Thus, the interquartile range may be 2 μm, with a median diameter of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 or 6.5 μm.

[0024] Thus, at least 30%, 40%, 50% or 60% of the microspheres may have diameters within the range 1.5-3.5 μm, 2.0-4.0 μm, 3.0-5.0 μm, 4.0-6.0 μm, 5.0-7.0 μm or 6.0-8.0 μm. Preferably a said percentage of the microspheres have diameters within a 1.0 μm range, such as 1.5-2.5 μm, 2.0-3.0 μm, 3.0-4.0 μm, 4.0-5.0 μm, 5.0-6.0 μm, 6.0-7.0 μm or 7.0-8.0 μm.

[0025] A further aspect of the invention provides hollow microspheres with proteinaceous walls in which at least 90%, preferably at least 95% or 99%, of the microspheres have a diameter in the range 1.0-8.0 μm; at least 90%, preferably at least 95% or 99%, of the microspheres have a wall thickness of 40-500 nm, preferably 100-500 nm.

[0026] Preferred aspects of the present invention will now be described by way of example and with reference to Figure 1, which is a partly cut away perspective view from the front and one side of suitable spray-drying apparatus for the process of the invention,

EXAMPLE 1

[0027] A suitable spray dryer (Figure 1) is available from A/S Niro Atomizer, Soeborg, Denmark under the trade designation "Mobile Minor". Details of its construction are given immediately before the claims herein. It comprises a centrifugal atomizer (Type M-02/B Minor), driven by an air turbine at an air pressure of min 4 bar and up to max 6 bar. At 6 bar an atomizer wheel speed of approx 33,000 rpm is reached. Turning on and off the compressed air to the atomizer is done by means of a valve placed in the instrument panel. The maximum consumption of compressed air to the atomizer is 17 Nm^3/h at a pressure of 6 bar. All parts coming into contact with the liquid feed and powder are made of stainless steel AISI 316, except for the pump feed tube and the atomizer wheel, which is made of stainless steel AISI 329, made to resist high centrifugal force.

[0028] The drying chamber has an inside made of stainless steel AISI 316, well insulated with Rockwool, and covered outside with a mild steel sheeting. The drying chamber is provided with a side light and observation pane for inspection during the operation. The roof of the drying chamber is made inside of stainless steel AISI 316 and outside of stainless steel AISI 304.

[0029] An air disperser made of stainless steel AISI 304 is used for distribution of the air in the drying chamber in order to achieve the best possible drying effect. An air duct, made of stainless steel AISI 316, provides lateral transportation of the exhaust air and the powder to the cyclone, which is made of stainless steel AISI 316 and designed to separate the powder and air.

[0030] A closing valve of the butterfly valve type, also made of stainless steel AISI 316 and having a gasket of silicone rubber, is used for powder discharge under the cyclone into a powder collecting glass jar tightly placed under the cyclone by means of a spring device.

[0031] A fan made of silumin, complete with 3-phase squirrel-cage motor, 0.25 kW, and V-belt drive with belt-guard, draws air and powder through the drying chamber and cyclone.

[0032] An air heater heats the drying air by means of electricity (total consumption 7.5 kWh/h, infinitely variable) and can give inlet air temperatures of up to about 350°C, although this is generally too high for preparing the microcapsules of the invention.

[0033] Equipment for two-fluid nozzle atomization may be added, which is made of stainless steel AISI 316, consisting of entrance pipe with nozzle holder and nozzle, to be placed in the ceiling of the drying chamber. The equipment includes an oil/water separator, reduction valve and pressure gauge for compressed air to the two-fluid nozzle. Consumption of compressed air: 8-15 kg/h at a pressure of 0.5-2.0 bar ($0.5\text{-}2.0 \times 10^5$ Pa).

[0034] A suitable feed pump for transport of wall-forming preparation feed to the atomizer device is a peristaltic pump. The pump is provided with a motor (1 x 220V, 50 Hz, 0.18 kW) and a continuously variable gear for manual adjustment. A feed pipe made of silicone hose leads from a feed tank (local supply) through the feed pump to the atomization device.

[0035] An absolute air filter, consisting of prefilter, filter body in stainless steel and absolute air filter, is used for the treatment of the ingoing drying air to render it completely clean.

[0036] A 20% solution of sterile, pyrogen-free rHA in pyrogen-free water (suitable for injection) was pumped to the nozzle of a two fluid nozzle atomiser mounted in the commercial spray drying unit described above. The peristaltic pump speed was maintained at a rate of approximately 10 ml/minute such that with an inlet air temperature of 220°C the outlet air temperature was maintained at 95°C.

[0037] Compressed air was supplied to the two fluid atomising nozzle at 2.0-6.0 Bar ($2.0\text{-}6.0 \times 10^5$Pa). In this range microcapsules with a mean size of 4.25-6.2 μm are obtained.

[0038] Typically an increase in mean particle size (by reduced atomisation pressure) led to an increase in the amount

of microcapsules over 10 μm in size (see Table 1).

TABLE 1

EFFECTS OF ATOMISATION PRESSURE ON FREQUENCY OF MICROCAPSULES OVER 10 μM IN DIAMETER	
Atomisation Pressure (x 10^5 Pa)	% Frequency over 10 μm
6.0	0.8
5.0	0.3
3.5	6.6
2.5	8.6
2.0	13.1

EXAMPLE 2

[0039] The process of Example 1 was repeated but with the following differences a centrifugal atomiser was used instead of a two fluid nozzle; the inlet temperature was 150°C (with the outlet air temperature still being sustained at 105°C); and compressed air was supplied to the nozzle at 1.0-6.0 x 10^5 Pa. The wheel rotated at 20-40,000 rpm and delivered droplets, and subsequently microcapsules, with a number mean diameter in the 1.0-8.0 μm range.

Further details of construction of atomiser

[0040] In Figure 1, reference numeral 1 denotes the feeding device. 2 is a ceiling air disperser which ensures effective control of the air flow pattern. Swirling air is directed around the vaned disc atomiser. 3 is a rotary atomiser or nozzle atomiser. 4 shows a stainless steel interconnecting pipe system which can easily be stripped down for cleaning. 5 are steps for access to the chamber top. 6 is the switch for an air valve for activation of the pneumatic lifting device when raising the chamber lid. 7 is a highly-efficient stainless steel cyclone in which the powder and the exhausted drying air are separated. 8 is a glass jar in which the powder is recovered. 9 is a centrally located instrument panel. 10 is a centrifugal exhaust fan with 3-phase motor. 11 is a damper for air flow control and 12 is an electric air heater which provides drying air temperatures up to 350°C. The drying air temperature can be continuously adjusted using a percentage timer switch. The maximum powder consumption is 7.5 kW.

Evaporative capacity			
Drying Air	Inlet Air Temperature	Outlet Air Temperature	Evaporative Capacity
85 kg/h	150°C	80°C	1,3 kg/h
85 kg/h	170°C	85°C	1,7 kg/h
80 kg/h	200°C	90°C	2,5 kg/h
80 kg/h	240°C	90°C	3,4 kg/h
75 kg/h	350°C	90°C	7,0 kg/h

Weight and dimension	
Weight	280 kgs
Length	1800 mm
Height	2200 mm
Width	925 mm

[0041] **Power.** The unit can only be operated on a 3-phase power supply (50 or 60 Hz) at alternative voltages of 440, 415, 400, 380, 220, 200 V.

[0042] All parts coming into contact with the liquid or the product are made of acid-resistant, stainless steel AISI 316.

Claims

1. A process for preparing hollow microcapsules, which comprises atomising a solution or dispersion of a wall-forming protein or polysaccharide material in a liquid carrier into a gas, to obtain the hollow microcapsules by evaporation of the liquid carrier, substantially without reducing the water-solubility of at least the outside of the microcapsules.

2. A process according to claim 1, wherein the wall-forming material is a protein.

3. A process according to claim 1, wherein the protein is collagen, gelatin or serum albumin.

4. A process according to claim 3, wherein the protein is human serum albumin, or an analogue or fragment thereof, prepared by recombinant DNA techniques.

5. A process according to any of claims 2 to 5, wherein the solution or dispersion comprises 10 to 30% of the protein.

6. Microcapsules obtainable by a process according to any of claims 1 to 5, of which more than 30% have a diameter within a 2 µm range and at least 90% have a diameter of 1 to 8 µm.

7. Microcapsules according to claim 6, of protein, which are 0.01-50.0 µm in diameter.

8. Microcapsules according to claim 6 or claim 7, which comprise 96-98% monomeric protein.

9. Microcapsules according to any of claims 6 to 8, in which the interquartile range of diameters is 2 µm or less and the median diameter is between 2 and 8 µm.

Patentansprüche

1. Verfahren zur Herstellung von hohlen Mikrokapseln, welches umfaßt, daß man eine Lösung oder Dispersion eines Wand-bildenden Protein- oder Polysaccharid-Materials in einem flüssigen Träger zu einem Gas zerstäubt, um die hohlen Mikrokapseln durch Verdampfen des flüssigen Trägers im wesentlichen ohne Verringerung der Wasserlöslichkeit zumindest der Außenseite der Mikrokapseln zu erhalten.

2. Verfahren nach Anspruch 1, in dem das Wand-bildende Material ein Protein ist.

3. Verfahren nach Anspruch 1, in dem das Protein Kollagen, Gelatine oder Serumalbumin ist.

4. Verfahren nach Anspruch 3, in dem das Protein Humanserumalbumin oder ein Analogon oder Fragment desselben ist, das durch rekombinante DNA-Techniken hergestellt wird.

5. Verfahren nach einem der Ansprüche 2 bis 5, in dem die Lösung oder Dispersion 10 bis 30 % des Proteins umfaßt.

6. Mikrokapseln, erhältlich durch ein Verfahren nach einem der Ansprüche 1 bis 5, von denen mehr als 30 % einen Durchmesser in einem 2µm-Bereich aufweisen und mindestens 90 % einen Durchmesser von 1 bis 8 µm aufweisen.

7. Mikrokapseln nach Anspruch 6 aus Protein, die einen Durchmesser von 0,01 bis 50,0 µm aufweisen.

8. Mikrokapseln nach Anspruch 6 oder 7, die 96 - 98 % monomeres Protein umfassen.

9. Mikrokapseln nach einem der Ansprüche 6 bis 8, worin die wahrscheinlichen Schwankung von Durchmessern 2 µm oder weniger beträgt und der Medianwert des Durchmessers zwischen 2 und 8µm liegt.

Revendications

1. Procédé pour préparer des microcapsules creuses qui comprend l'atomisation dans un gaz d'une solution ou dispersion d'un matériau protéique ou polysaccharidique formant des parois dans un support liquide pour obtenir

les microcapsules creuses par évaporation du support liquide, sensiblement sans réduire la solubilité dans l'eau d'au moins le côté extérieur des microcapsules.

2. Procédé selon la revendication 1 où le matériau formant des parois est une protéine.

3. Procédé selon la revendication 1 où la protéine est le collagène, la gélatine ou la sérumalbumine.

4. Procédé selon la revendication 3 où la protéine est la sérumalbumine humaine, ou un analogue ou fragment de celle-ci, préparée par des techniques de recombinaison d'ADN.

5. Procédé selon l'une quelconque des revendications 2 à 5 où la solution ou dispersion comprend 10 à 30 % de protéine.

6. Microcapsules pouvant être obtenues par un procédé selon l'une quelconque des revendications 1 à 5 dont plus de 30 % ont un diamètre dans un domaine de 2 µm et au moins 90 % ont un diamètre de 1 à 8 µm.

7. Microcapsules selon la revendication 6, de protéine, qui ont un diamètre de 0,01-50,0 µm.

8. Microcapsules selon la revendication 6 ou la revendication 7 qui comprennent 96-98 % de protéine monomère.

9. Microcapsules selon l'une quelconque des revendications 6 à 8 dans lesquelles l'écart interquartile des diamètres est 2 µm ou moins et le diamètre médian est de 2 à 8 µm.

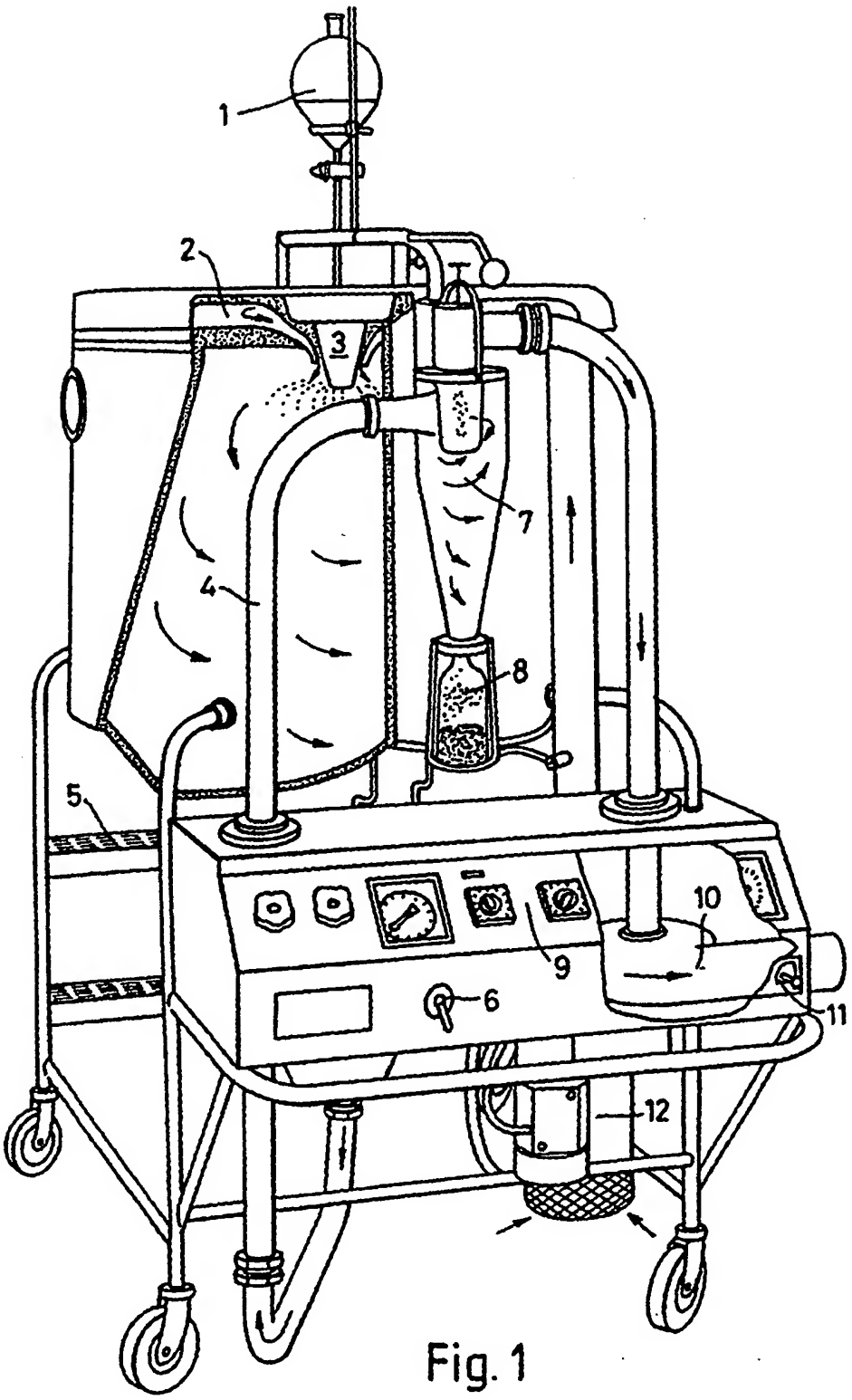

Fig. 1